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(71) Applicant: DAKO A/S [DK/DK]; Produktionsvej 42, DK-2600 Glostrup (DK).

(72) Inventors: HYLDIG-NIELSEN, Jens, Jørgen; Klingseyvej 16, DK-2720 Vanløse (DK). PLUZEK, Karl-Johan; Primulahaven 38, DK-2765 Smørum (DK).

(74) Agent: FINK, Kirsten; Dako A/S, Produktionsvej 42, DK-2600 Glostrup (DK).

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(54) Title: POLYCLONAL ANTIBODY TO PNA/NUCLEIC ACID COMPLEXES

(57) Abstract

This invention relates to polyclonal antibodies to complexes formed between PNA (Peptide Nucleic Acid) and nucleic acids, particularly polyclonal antibodies to PNA/DNA or PNA/RNA complexes. The preferred polyclonal antibodies to PNA/DNA or PNA/RNA complexes do not bind to single-stranded PNA, double-stranded nucleic acid or single-stranded nucleic acid. Peptide Nucleic Acids (PNA) are newly developed, not naturally occurring compounds comprising a polyamide backbone bearing a plurality of ligands such as naturally occurring nucleobases attached to a polyamide backbone through a suitable linker. PNA oligomers with a backbone of N-(2-aminoethyl)-glycin units have a surprising high affinity for complementary nucleic acid forming very stable and specific complexes. This property makes PNA oligomers suitable as hybridization probes for detection of nucleic acids. The usability of PNA as hybridization probes is greatly increased by the present antibodies. The antibodies according to the invention are useful in the capture, recognition, detection, identification or quantitation of nucleic acids in biological samples, via their ability to react with PNA-nucleic acid complexes.

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Polyclonal antibody to PNA/nucleic acid complexes.

This invention relates to polyclonal antibodies to complexes formed between PNA (Peptide Nucleic Acid) and nucleic acids.

Peptide Nucleic Acids (PNA) are newly developed, not naturally occurring compounds comprising a polyamide backbone bearing a plurality of ligands such as naturally occuring nucleobases attached to an amide backbone through a suitable linker. PNA oligomers have a surprising high affinity for complementary nucleic acid forming very stable and specific complexes. This property makes such PNA oligomers suitable as hybridization probes for detection of nucleic acids. The usability of PNA as hybridization probes is greatly increased by the present antibodies.

The antibodies according to the invention are useful in the capture, recognition, detection, identification or quantitation of nucleic acids in biological samples, via their ability to react with PNA-nucleic acid complexes.

Background of the invention

The capture, recognition, detection, identification or quantitation of one or more chemical or biological entities is useful in the fields of recombinant DNA, human and veterinary medicine, agriculture and food science, among others. In particular, these technique can be used to detect and identify etiological agents such as bacteria and vira, to screen bacteria for antibiotic resistance, to aid in the diagnosis of genetic disorders and to detect cancerous cells.

The state-of-the-art nucleic acid hybridization assay techniques generally involve hybridization with a labelled form of a complementary polynucleotide probe. Hybridization between particular base sequences or genes of interest in

the sample nucleic acid and labelled probe is determined by detection of the labelled hybrids. The preparation of labelled probe generally involves the enzymatic incorporation of radiolabelled or modified nucleotides or chemical modification of the probe to attach or form a detectable chemical group. Preparation of labelled probes is often time consuming and expensive and has to be carried out without destroying the ability of the probe to detectably hybridize with its complementary sequence.

Reagents for directly detecting the polynucleotide duplex formed as a result of hybridization between the sample and probe and thereby avoid the chemical labelling of the probes, would overcome this problem.

The generation of specific polyclonal antibodies that will bind double-stranded nucleic acid but not single-stranded nucleic acid is complicated by the fact that polyclonal antisera may contain antibodies that will cross-react with single-stranded nucleic acid. Polyclonal antisera may also contain naturally occuring antibodies to single-stranded nucleic acid or antibodies to single-stranded nucleic acid arising as a result of the immunization.

Monoclonal antibody technology can provide a means to select an antibody with desired affinity and specificity which will overcome the problems discussed above. Such monoclonal antibodies which will selectively bind double-stranded DNA (US 4,623,627) or DNA-RNA hybrids (US 4,833,084) have been prepared and used in the detection of duplexes formed between particular base sequences of interest in the sample nucleic acid and a probe with a known complementary sequence.

A new alternative arising from the construction of the nucleic acid analoque PNA (Peptide Nucleic Acid), is to use a PNA oligomer as a detection probe and generate antibodies that bind to PNA-nucleic acid complexes.

Peptide Nucleic Acids (PNAs) are described in WO 92/20702 as compounds

comprising a polyamide backbone bearing a plurality of ligands such as naturally occuring nucleobases attached to a polyamide backbone through a suitable linker. It has recently been shown that PNA in which the backbone is structurally homomorphous with the deoxyribose backbone and consists of N-(2-aminoethyl)glycin units to which the nucleobases are attached can hybridize to complementary oligonucleotides to form PNA-nucleic acid complexes (Egholm et al., Nature, vol 365, 566-568 (1993)).

SUMMARY OF THE INVENTION

One aspect of the present invention is polyclonal antibodies that binds to complexes formed between PNA and nucleic acids.

Apart from sharing the feature of base pairing, PNA/nucleic acid complexes and nucleic acid duplexes, such as DNA/DNA or DNA/RNA duplexes, possess substantially different properties in that the PNA of a PNA/nucleic acid complex has a backbone consisting of an N-(2-aminoethyl)glycine oligomer or polymer which is achiral and non charged as opposed to the corresponding strands of an nucleic acid duplex, wherein the backbone is a sequence of nucleotides conyaining one anion for each phosphate group. The ensuing steric and conformational differences between the two types of compounds makes it absolutely unpredictable whether antibodies binding specifically to PNA/nucleic acid complexes could be made available.

Other aspects of the invention are polyclonal antibodies that binds to complexes formed between PNA and DNA or PNA and RNA.

In preferred embodiments the polyclonal antibodies to complexes formed between PNA and nucleic acids do not bind to single-stranded PNA, double-stranded nucleic acid or single-stranded nucleic acid.

In one of these embodiments the antibody binds to a complex formed between PNA and DNA, but not to PNA/RNA complexes, double-stranded DNA, DNA/RNA hybrids, single-stranded PNA or single-stranded nucleic acid.

In another of these embodiments the antibody binds to a complex formed between PNA and RNA, but not to PNA/DNA complexes, double-stranded DNA, DNA/RNA hybrids, single-stranded PNA or single-stranded nucleic acid.

Polyclonal antibodies that binds to PNA/nucleic acid complexes irrespective of the base sequence are also part of the invention.

Polyclonal antibodies of the present invention can be obtained by immunizing a host animal with a complex formed by contacting PNA with a nucleic acid, particularly a complex formed between PNA with a backbone of N-(2-amino-ethyl)glycin units and DNA or RNA.

Various methods for detecting a particular nucleic acid sequence in a test sample are additional aspects of the invention, whereby the antibodies according to the invention can be used in the capture, recognition, detection, identification or quantitation of one or more chemical or biological entities.

The antibodies are very useful in the human and veterinary field. Especially contemplated is the use of the present antibodies to detect the presence or amount of infectious agents in humans such as chlamydial or gonococcal organisms or infections with Epstein Barr virus or papillomavirus. The present antibodies are also useful in the general field of cytogenetics such as chromosomal painting.

The invention also provides a kit containing antibody according to the invention, which antibody might be in a detectably labelled form, a PNA sequence that is complementary to the nucleic acid sequence to be detected and a detection system.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

When referring to the antibody of the present invention it is intended to include whole, intact antibodies, antibody fragments, polyfunctional antibody aggregates, or in general any antibody-derived substance that comprises at least one antibody combining site having the characteristics described herein. Antibodies of any of the known classes and subclasses of immunoglobulins are contemplated, e.g., IgG, IgM, and so forth, as well as active fragments such as the Ig fragments conventionally known as scFv, Fab, F(ab'), and $F(ab')_2$.

The term nucleic acid covers a nucleotide polymer composed of subunits, which are either deoxyribonucleosides or ribonucleosides joined together by phosphodiester bridges between the 5'-position of one nucleoside and the 3'-position of another nucleoside. They may be DNA or various types of RNA. The terms bases and nucleobases are used interchangeable for pyrimidine and purine bases of the nucleic acids and PNA.

The PNAs are synthesized according to the procedure described in "Improved Synthesis, Purification and Characterization of PNA Oligomers", Presented at the 3rd Solid-Phase Symposium, Oxford UK, Aug. 31 - Sept. 4, 1994.

The PNA-nucleic acid complex used for immunization of an animal according to the present invention might comprise PNA and DNA or RNA. Since both nucleic acid and PNA are devoid of diversed peptide sources, both nucleic acid duplexes and PNA/nucleic acid complexes would be expected to be essentially non-immunogenic in normal host animals (i.e. animal which are not prone to generate auto-antibodies against nucleic acid) when injected <u>per se</u>. Whereas in these circumstances the conventional conjugation of the non-immunogenic antigen to a carrier foreign to the host animal generally has been found to be impractical and laborious and furthermore induce a risk of creating structural changes in the antigen, an immune response towards nucleic acid duplexes has been elicited by immunizing normal host animals

with non-covalent, ionic complexes formed between the poly-anionic nucleic acid duplex and a poly-cationic protein derivative, particular a methylated albumin or globulin species (US 4,623,627, US 4,833,084). According to the present invention it has now surprisingly been found that antibodies against PNA/DNA complexes can be raised by immunizing a normal host animal with a mixture comprising a PNA/DNA complex and a non-derivatized protein heterologous to the host animal, such as ovalbumin. This technology can be applied to immunization with PNA/RNA complexes.

A PNA-DNA complex can be prepared by contacting double-stranded or single-stranded DNA with a PNA molecule having a base sequence that is complementary to all or part of the DNA sequence, heating the mixture to form single-stranded molecules and allowing the mixture to cool slowly to room temperature. A PNA-RNA complex can be prepared by contacting RNA with a PNA molecule having a base sequence that is complementary to all or part of the RNA sequence, heating the mixture and allowing the mixture to cool slowly to room temperature. A suitable quantity of one of the PNA-nucleic acid complexes is mixed with an adjuvant. The immunogen might be used unconjugated or conjugated to a suitable carrier such as KLH (Keyhole Limpet Hemocyanin), ovalbumin and dextrans.

Polyclonal antibodies of the present invention were obtained by immunizing rabbits with a mixture of a PNA/DNA complex, wherein the PNA had a N-(2-aminoethyl)glycin backbone, ovalbumin and a suitable adjuvant. The immunization schedule and bleeding were otherwise performed as described by Harboe and Ingild, Scand. J. Immunol., vol 17, Suppl. 10, 345-351, 1983. Polyclonal antibodies with a high specificity for PNA/DNA complexes were obtained from the serum of the immunized rabbits. Many animals are suitable as host animals for the production of polyclonal antibodies.

As will appear from table 1 the polyclonal antibodies raised against a complex of PNA and a 45-mer DNA reacted strongly with PNA/DNA and PNA/RNA

complexes and not with double-stranded DNA, DNA/RNA-hybrids, single-stranded DNA or single-stranded PNA. The antibody reacted strongly with four different PNA/DNA complexes wherein the base sequence was different indicating that the antibodies recognize the conformation of the PNA/DNA complex rather than any specific base sequence in the PNA or DNA.

Polyclonal antibodies of the present invention can be obtained by immunizing rabbits with a mixture of a PNA/RNA complex, wherein the PNA had a N-(2-aminoethyl)glycin backbone, ovalbumin and a suitable adjuvant. The immunization schedule and bleeding are otherwise performed as described by Harboe and Ingild, Scand. J. Immunol., vol 17, Suppl. 10, 345-351, 1983. Many animals are suitable as host animals for the production of polyclonal antibodies.

The antibodies of the present invention are characterized by a high degree of specificity for PNA-nucleic acid complexes. They do not to any significant degree bind to double-stranded nucleic acid, single-stranded PNA or single-stranded nucleic acid. The specificity of the epitope(s) recognized by the present antibodies appears to be dictated by the conformation of the PNA-nucleic acid complex rather than by any specific sequence of the PNA or the nucleic acid.

A high specificity and affinity of the antibodies according to the invention give significant advantages when used in the isolation, detection and quantitation of PNA-nucleic acid complexes formed between PNA and nucleic acid to be detected in a biological sample. Thus the antibodies with a high specificity for PNA/DNA complexes are particularly valuable in DNA probe based analysis for identifying infectious agents in humans such as chlamydial or gonoccal organims. These antibodies are also very useful in the general field of cytogenetics such as chromosome painting.

Antibodies according to the invention having a high specificity and affinity for

PNA/RNA complexes are particularly useful in RNA probe based analysis, for example for identifying mRNA or rRNA sequences specific for particular organisms.

Depending on the particular use of the antibody according to the invention the antibody may be coupled with a detectable label such as enzymatically active groups like coenzymes, enzyme inhibitors and enzymes themselves, fluorescers, chromophores, luminescers, specifically bindable ligands such as biotin or haptens.

The antibodies according to the invention are valuable tools in a number of different methods for detecting a particular nucleic acid sequence, such as a method comprising

(a) forming a complex between the particular nucleic acid sequence to be detected in the sample and a sequence of PNA that is complementary to the nucleic acid sequence to be detected, the complex having at least one epitope for an antibody according to the invention,

(b) contacting any complex that is formed between the PNA sequence and the nucleic acid sequence to be detected with an antibody according to the invention, and

(c) determining the presence of antibody-PNA-nucleic acid complexes.

In order to be able to catch the antibody-PNA-nucleic acid complex in (c), the PNA sequence in (a) can be immobilized to a solid phase prior to the contact with the nucleic acid sequence to be detected or the antibody used in (b) can be immobilized to a solid phase prior to contact with the PNA-nucleic acid complex .

If the nucleic acid sequences to be detected exists in an immobilized state in a biological specimen, a method comprising

(a) forming a complex between the particular nucleic acid sequence to be detected in the specimen and a sequence of PNA that is complementary to the nucleic acid sequence to be detected, the complex having at least one epitope for an antibody according to the invention,

(b) contacting any complex that is formed between the PNA sequence and the nucleic acid sequence to be detected with an antibody according to the invention, and

(c) determining the presence of antibody-PNA-nucleic acid complexes,

can be used.

In another method the initial step could be an immobilization of the nucleic acid sequence to be detected in a method comprising

(a) immobilizing the nucleic acid sequence to be detected to a solid phase,

(b) forming a complex between the particular nucleic acid sequence to be detected in the sample and a sequence of PNA that is complementary to the nucleic acid sequence to be detected, the complex having at least one epitope for an antibody according to the invention,

(c) contacting any complex that is formed between the PNA sequence and the nucleic acid sequence to be detected with an antibody according to the invention, and

(d) determining the presence of antibody-PNA-nucleic acid complexes.

Particularly attractive applications of the present antibodies are described below.

A kit for carrying out the described methods or other methods taking advantage of the antibodies according to the present invention contains in addition to the present antibody in labelled or unlabelled form, a PNA sequence that is complementary to the nucleotide sequence to be detected, i.e. a PNA probe, and a detection system. The detection system may comprise an enzyme which is able to react with a substrate to form a coloured soluble or insoluble reaction product.

<u>Application 1:</u> <u>Hybridization and detection in solution.</u>

A polynucleotide sequence of interest can be determined in solution by contact with PNA molecules complementary to the sequence of interest followed by contact with an antibody according to the present invention, recognizing the PNA-nucleic acid complexes but not free PNA or nucleic acids. These reactions will result in a large complex which may be detected e.g. in a turbidimetric assay format.

<u>Application 2:</u> <u>Solution hybridization and detection after immobilization.</u>

A polynucleotide sequence of interest can be determined by contacting it with a PNA-oligomer complementary to the sequence of interest. The complexes formed are, while still in solution, contacted with an antibody according to the present invention in a labelled or unlabelled form. The PNA-nucleic acid-antibody complex formed is then captured using e.g. an antibody according to the present invention which has been immobilized on a solid phase. Unbound materials is washed off and the amount of bound PNA-nucleic acid-antibody

complex is determined either via the label on the antibody or by using a secondary antibody detection system, provided that the immobilized antibody is derived from an alternative species from that of the detecting antibody.

Alternatively, the PNA-oligomers complementary to the sequence of interest may be labelled with a moiety e.g. biotin, fluorescein, or other haptens which is suitable for catching of PNA-nucleic acid complexes. Unbound materials is washed off and the amount of bound PNA-nucleic acid-antibody complex is determined either via a label on the antibody or by using a secondary antibody detection system.

Application 3: Capture assay

A traditional capture assay consists of the following steps recognition, capture, detection. Such assays may be build up in many different ways. One particular interesting example is outlined below.

An antibody capable of binding a PNA-nucleic acid complex is coupled or alternative immobilized to a solid support, e.g. an ELISA plate. PNA oligomers and sample is mixed and allowed to react in solution in the wells of the ELISA-plate. If complexes between the PNA and the sample nucleic acids are formed, these complexes are captured by the immobilized antibody. Unbound materials are washed off, and to ensure available binding sites for the detection system PNA-oligomers can be added and allowed to react with the bound nucleic acids. An antibody of this invention, e.g. conjugated with an enzyme, is added and allowed to react with the formed PNA-nucleic acid complexes. After washing a suitable enzyme substrate is added and the amount of bound materials is measured.

It might be possible to perform two or more of the steps indicated above simultaneously. Also it might be possible to built up either the capture or the detection step based on other recognizable moieties than the PNA-nucleic

acid complex indicated above. Such moities could e.g. be biotinylated PNA-oligomers or PNA-oligomers labelled with other haptens, peptides,or polypeptides.

Application 4: Detection of PNA/nucleic acid complexes on solid phase.

Complexes formed between a PNA-oligomer and nucleic acids in which either the PNA or the nucleic acid initially was immobilized on a solid phase can be detected by the antibody of the present invention. This detection can be performed either directly using an antibody conjugated to an enzyme, a fluorescent marker or an other signal generating system, or indirectly using one of the secondary detection systems commonly used for detecting antibodies bound to their target. The solid phase considered should be understood in a very broad sense like e.g. nylon or nitrocellulose membranes (Southern or Northern blots), a tissue section (*in situ* hybridization), or a plastic surface (an ELISA format).

This system has the advantage that the normally very extensive washing procedures included in these technologies can be reduced to a minimum as unspecific bound PNA-oligomers, being single-stranded, will not give rise to a signal as the antibody only recognizes PNA hybridized to nucleic acids. For the same reason this type of analysis will result in less problems with background caused by unspecific binding of the PNA-oligomer.

Application 5: Biosensor systems

One example of dynamic reaction detection using a biosensor surface is the surface plasmon resonance (SPR) detection system, e.g. employed by the BIAcore biosensor system (Pharmacia). The interaction of biomolecules with an immobilized ligand on a sensor chip is measured at the surface using evanescent light. The system includes a sensor chip to which the ligand can be immobilized in a hydrophilic dextran matrix, a miniaturised fluidics cartridge for the transport of analytes and reagents to the sensor surface, a SPR detec-

tor, an autosampler and system control and evaluation software. Specific ligands are covalently immobilized to the sensor chip through amine, thiol or aldehyde chemistry or biospecifically by e.g. biotin - avidin interaction.

The antibody of this invention is coupled to the dextran layer of a sensor chip used in the BIAcore biosensor-system (or other types of biosensor systems). A sample is mixed with a PNA-oligomer and incubated so that a complex is formed between PNA and sample nucleic acids complementary to the PNA-oligomer used. The sample is passed through the flow system of the BIAcore and the antibody coupled to the dextran surface will bind the PNA-nucleic acid complexes if such complexes have been formed. Based on the SPR detection employed by the BIAcore this binding will generate a signal dependent on the amount of materials bound to the surface.

Application 6: Detection of bound PNA in cells.

Under suitable conditions, PNA oligomers may be able to penetrate the cell-wall of living or fixed cells, e.g. cell-lines, hemopoetic cells, and animal/human tissues (important in therapeutic applications). It will be important to be able to detect the PNA-oligomers that have reacted with their different targets in the individual cells. Labelling with haptens or other reporter molecules of the PNA-oligomer will not be advantageous as this will inhibit (interfere with) the penetration into the cells. Of great significans is the detection of reacted PNA-oligomers, either by immunohistochemistry (in frozen or fixed tissue biopsies) or by Flow-cytometry (e.g. on cells treated with detergent, acetone or alcohol), or in an in vivo set up to detect binding and/or tissue distribution of PNA's added to a cell culture or administered to a living animal.

The following examples illustrate various aspects of the invention. These examples are not intended to limit the invention in any way.

EXAMPLES

Example 1. Preparation of PNA/DNA complexes.

PNA-oligomers, molecules in which the backbone is structurally homomorphous with the deoxyribose backbone of DNA and which consists of N-(2-aminoethyl)glycine units to which nucleobases are attached through a methylenecarbonyl linker, were synthesized and purified as described in "Improved Synthesis, Purification and characterization of PNA Oligomers", presented at the 3rd Solid-Phase Symposium, Oxford UK, Aug. 31 - Sept. 4, 1994, and by M. Egholm et al., J. Am. Chem. Soc. 114, 1895-1897 (1992) and M. Egholm et al.,J. Chem. Soc. chem. Commun. 800-801 (1993). The base sequence of the PNA used is preferably virtually non-self-complementary in order to avoid self-hybridization in the PNA molecule. The number of purines and pyrimidines is approximately equal to allow formation of a double helix configuration rather than a tripple helix configuration.

DNA oligomers were synthesized on an abi 381A DNA synthesizer from Applied Biosystems using a standard 381A cycle/procedure . The monomers used were standard ß-cyanoethyl phosphoamidites for Applied Biosystems Synthesizer.

The antigen used for immunizing rabbits was made adding one mol of a 45-mer synthetic polydeoxyribonucleotide (DNA) and three mols of a 15-mer PNA oligomer. The 45-mer polydeoxyribonucleotide was designed as three repeated units of 15 nucleotides and the 15-mer PNA oligomer had a base sequence complementary to the base sequence of the 15-mer unit of the polydeoxyribonucleotide.

The base sequence of the 45-mer polydeoxyribonucleotide was as follows: 5'-GCA AAT GCT CTA GGC GCA AAT GCT CTA GGC GCA AAT GCT CTA GGC-3'.

The base sequence of the 15-mer PNA oligomer was as follows:
5'-H-GCC TAG AGC ATT TGC-NH_2-3'

Antigen for immunization of rabbits was prepared by mixing the following in a total volume of 6.01 mL:

106 OD_{260} 45-mer polydeoxyribonucleotide (DNA)
106 OD_{260} 15-mer PNA oligomer
50 mM Tris-HCl, pH 7.5
50 mM NaCl

(Note: A ratio of 1 OD of the 45-mer DNA to 1 OD of the 15-mer PNA oligomer is equivalent to a molar ratio of approximately 1:3 using the same extinction coefficient for DNA and PNA.)

The mixture was heated to 92°C in a heating block and allowed to cool slowly to room temperature. This solution was evaporated in a vacuum centrifuge and resuspended in 600 µL H_2O. Final concentration of PNA/DNA hybrid was approximately 10 mg/mL.

Complex formation was characterized by electrophoresis of Biotin labelled PNA/DNA complexes in 20% polyacrylamide gels (TBE-buffer, 89 mM Tris-borate, 2 mM EDTA) followed by transfer to a nitrocellulose membrane and visualization using an Alkaline Phosphatase (AP) conjugated Streptavidin complex and the substrate NBT/BCIP.

<u>Example 2</u> <u>Test systems.</u>
Identification of specific antibody activity were based on results obtained in different ELISA formats. Microtiter plates were coated with streptavidin followed by blocking of excess binding sites. The complexes/compounds used for testing the specificity were labelled with biotin.

PNA oligomers labelled with biotin are produced by using the "solid phase" principle for Boc synthesis. A linker comprising two units of 2-(aminoethoxy)ethoxy acetic acid (AEEA) is attached to the PNA oligomer on the resin (see above), and biotin is attached in the following way. Two solutions were used. The first solution contained 0.1 M biotin in 5% s-collidin in DMF with 2 equivalents of N-ethyldicyclohexylamine and the second solution contained 0.18 M HBTU (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) in DMF. The two solutions were mixed in a ratio of 2 to 1 and the mixture was left for approximately one minute before it was combined with the resin to which the PNA oligomer with two units of AEEA were attached.

For biotin labelling of DNA the following two procedures were used. For labelling in the 5' end of the DNA oligomers a spacer (Spacer phosphoramidite, Clontech Laboratories) was connected to the 5'-OH of the oligomer and then reacted with a biotin labelling reagent (Biotin CE phosphoramidite,22-0001-35, Cruachem Limited). For labelling in the 3' end of the oligomers the DNA synthesis was started from a Biotin-CPG support (3'-Biotin-ON CPG cat # RP-5225-2 K.J. Ross Petersen, Agern Alle 3, DK-2970 Hørsholm). The first reagent was a spacer (Spacer phosphoamidite, Clontech Laboratories, Inc.) and the monomer reagents were added for synthesising the oligomer.

RNA oligomer were purchased from "DNA Technology Aps, Science Park Aarhus, Gustav Wieds vej 10, DK-8000 Aarhus.

The following detection systems were used:

1. layer:	streptavidin
2. layer:	100 µl biotinylated test complex/compound (100 ng/mL)
3. layer:	dilution of the polyclonal antibody according to the invention
4. layer:	anti rabbit antibodies conjugated with HRP (Horseradish peroxidase)
Substrate:	OPD (o-phenylenediamine)

The following test complexes/compounds were used:

A. A PNA/DNA complex comprising 45-mer DNA and 3 units of 15-mer PNA having the same base sequence as the complex used for immunization and wherein biotin is attached to the 3' end of the DNA as described above.

B. A single stranded 15-mer DNA (ssDNA) corresponding to one of the three 15 nucleotide long sequences of the 45-mer DNA in the complex used for immunization and labelled with biotin at the 5' end as described above.

C. A single stranded 15-mer PNA (ssPNA) with a base sequence corresponding to the 15-mer PNA in the complex used for immunization and labelled with biotin in the 5' end as descibed above.

D. A PNA/DNA complex comprising a 20-mer PNA and a 20-mer DNA having a base sequence that is different from the sequence of the complex used for immunization and wherein the PNA is labelled with biotin at the 5' end as described above. The sequence used was:
PNA: 5'-Bio-AEEA-AEEA-CGG-CCG-CCG-ATA-TTG-GCA-AC-NH_2-3'
DNA: 5'-GTT-GCC-AAT-ATC-GGC-GGC-CG-3'

E. A single stranded 15-mer PNA (ssPNA) with a base sequence as described in D, i.e. different from the base sequence used for immunization.

F. A PNA/DNA complex comprising a 17-mer PNA and DNA wherein the base sequence is different from the sequence of PNA/DNA in A and D and wherein the complexes were labelled with bitoin either at the 5' end of the DNA (F1) or at the 5' end of the PNA (F2). The following sequences were used:

F1: DNA: 5'-Bio-spacer-ATT-GTT-TCG-GCA-ATT-GT-3'
PNA: 5'-H-AEEA-ACA-ATT-GCC-GAA-ACA-AT-NH_2-3'

F2: DNA: 5'-ATT-GTT-TCG-GCA-ATT-GT-3'
PNA: 5'-Bio-AEEA-ACA-ATT-GCC-GAA-ACA-AT-NH_2-3'

G. Single stranded DNA with the base sequence described in F1 above.

H. A PNA/RNA complex comprising a 19-mer PNA and a 19-mer RNA.

I Double stranded DNA comprising fragments of calf thymus DNA (Sigma D-1501; converted to fragments comprising from 200 to 1000 bp).

J A DNA/RNA duplex comprising the 19-mer RNA in H and the complementary DNA sequence.

Example 3. Production of polyclonal antibodies.

To approimately 5 mg of the PNA/DNA antigen described in example 1 (final concentration approximately 10 mg/mL) was added 0.1 mL ovalbumin solution (10 mg Sigma A-7641 lot 70H8210 per mL of 0.1 M NaCl, 0.015 M NaN_3) and 0.01 mL 1.5 M NaN_3 and this mixture was stepwise whirl mixed into 0.5 mL Titermax #R1 adjuvant (Vaxcel Inc., Norcross, Georgia, USA). Before immunization the volume of this immunization composition was either approximately 1 mL (the first four immunizations) or adjusted to 2 mL (the following immunizations) by addition and mixing with 0.1 M NaCl, 0.015 M NaN_3.

Five rabbits were each immunized subcutaneously with a dose of approximately 0.5 mg PNA/DNA (total) per immunization, by injecting a volume of 0.1 mL immunization composition for the first four immunizations and 0.2 mL for the following immunizations. Blood samples were taken before immunization and samples for analysis of antibody activity were taken 8 and 10 weeks after the first immunization. The immunization schedule and bleeding were as

described in Harboe and Ingild A, Scand. J. Immunol., vol 17, Suppl. 10, 345-351, 1983.

Sera taken before immunization and 10 weeks after immunization of five rabbits were analyzed in the test systems described in example 2. The sera were diluted 2 fold starting at 1:250 (1:250, 1:500, 1:1000, 1:2000, 1:4000, 1:8000, 1:16000, 1:32000) and horseradish peroxidase conjugated swine anti rabbit immunoglobulin was used for visualization. In table 1 the results from one representative experiment are shown. The optical density at 492 nm gained by diluting the antibodies 1:500 is shown in tests of sera using the test complexes/compounds A, B, C, D, E, F, G, H, I and J described in example 2.

TABLE 1

Test complex/compounds	Serum taken before immunization	Serum taken 10 weeks after first immunization
A (PNA/DNA)	0.257	> 3.0
B (DNA)	0.155	0.437
C (PNA)	0.179	0.317
D (PNA/DNA)	0.115	2.467
E (PNA)	0.248	0.649
F1 (PNA/DNA)	0.200	2.751
F2 (PNA/DNA)	0.205	2.456
G (DNA)	0.088	0.133
H (PNA/RNA)	0.139	2.011
I (dsDNA)	0.129	0.086
J (RNA/DNA)	0.106	0.107

As shown in table 1, serum taken from the bleeding 10 weeks after immunization strongly reacted with the PNA/DNA complexes (A, D, F1 and F2) and the PNA/RNA complex (H), whereas no significant reaction was seen using the serum taken before immunization. Dose response curves were obtained when testing the dilutions of the serum 10 weeks after immunization using these antigens. No significant reactions was seen when testing the sera on any of the other antigens mentioned above. Thus the polyclonal antibody reacts with PNA/nucleic acid and this reaction appears to be independent of the base sequence.

Sera was also testet in a filter dot assay using the test complexes/compounds A, H and J described in example 2 and the individual PNA, DNA and RNA strnds included in these complexes. Also two preparations of dsDNA, one consisting of two complementary oligonucleotides and a plasmid DNA, were analysized in this dot blot assay. The test compounds were dotted in amounts of 20, 10, 5 or 2.5 ng on filters, followed by sera diluted 1:2000 in 0.5% casein in TS-buffer (50 mM Tris-HCl, 500 mM NaCl, pH 9.0) and the antibody captured by the test compounds on the filters was visualized with alkaline phosphatase conjugated goat anti rabbit Ig. Only positive reaction was seen with the PNA/DNA complex A in example 2 at a detection limit of 5 ng.

Sera was tested in a Southern blot format. Three sets of four different DNA oligonucleotides were loaded on a denaturing 20% polyacrylamide gel. Two of the DNA oligonucleotides used are as described in A and F1 in example 2 and the other two were unrelated DNA oligomers. After electrophoresis the DNA oligonucleotides were transferred to Nytran, nitrocellulose membranes. One filter was hybridized with the PNA oligomer described in A (from example 2), one filter with the PNA oligomer described in F1 (example 2) and the last filter with a mixture of these two PNA oligomers. After blocking unreacted sites, the membranes were incubated 2 hours at 30 °C with the polyclonal antibody according to the invention (diluted 1:2000). Bound antibody was visualized using alkaline phosphatase conjugated goat anti rabbit Ig and the

substrate NBT/BCIP. Only the lanes containing DNA oligonucleotides complementary to the PNA oligomer used for hybridization gave rise to a band with a position as expected for the size of the DNA oligonucleotides used. Thus the polyclonal antibody according to the invention recognizes the PNA/DNA complexes formed in a Southern blot on the nitrocellulose membranes.

Although PNA comprising a N-(2-aminoethyl)glycin backbone is the preferred type of PNA, this should not be taken as a limitation. It is expected that PNA with other types of backbone can be used in a similar way as long as the PNA is capable of forming stable complexes with nucleic acids.

Modification of the above described modes for carrying out the invention that will be clear to those skilled in the fields of immunochemistry, nucleic acid chemistry and related fields are intended to be within the scope of the following claims.

Claims.

1. Polyclonal antibody characterized in that it binds to complexes formed between PNA (Peptide Nucleic Acid) and nucleic acids.

2. Antibody according to claim 1, characterized in that it does not bind to single-stranded PNA, double-stranded nucleic acid, or single-stranded nucleic acid.

3. Antibody according to claim 2, characterized in that it binds to a complex formed between PNA and DNA, but not to PNA/RNA complexes, double-stranded DNA, DNA/RNA-hybrids, single-stranded PNA or single-stranded nucleic acid.

4. Antibody according to any of the claims 1 to 3, characterized in that it is obtainable by immunizing a host animal with a complex formed between PNA with a N-(2-aminoethyl)glycin backbone and DNA.

5. Antibody according to any of the claims 1 to 4, characterized in that it binds to PNA/DNA complexes irrespective of the base sequence.

6. Antibody according to claim 1 or 2, characterized in that it is obtainable by immunizing a host animal with a complex formed between PNA with a N-(2-aminoethyl)glycin backbone and RNA.

7. Antibody according to any of the claims 1 to 6 in detectably labelled form.

8. A method for detecting a particular nucleic acid sequence in a test sample, comprising

(a) forming a complex between the particular nucleic acid sequence to be detected in the sample and a sequence of PNA that is complementary to the nucleic acid sequence to be detected, the complex having at least one epitope for an antibody according to any of the claims 1 to 7,

(b) contacting any complex that is formed between the PNA sequence and the nucleic acid sequence to be detected with an antibody according to any of the claims 1 to 7, and

(c) determining the presence of antibody-PNA-nucleic acid complexes.

9. A method according to claim 8, characterized in that the antibody used in (b) is immobilized to a solid phase prior to contact with the PNA-nucleic acid complex.

10. A method according to claim 8, characterized in that the PNA sequence in (a) is immobilized to a solid phase prior to the contact with the nucleic acid sequence to be detected.

11. A method for detecting a particular nucleic acid sequence which exists in an immobilized state in a biological specimen, comprising

(a) forming a complex between the particular nucleic acid sequence to be detected in the specimen and a sequence of PNA that is complementary to the nucleic acid sequence to be detected, the complex having at least one epitope for an antibody according to any of the claims 1 to 7,

(b) contacting any complex that is formed between the PNA

sequence and the nucleic acid sequence to be detected with an antibody according to any of the claims 1 to 7, and

(c) determining the presence of antibody-PNA-nucleic acid complexes.

12. A method for detecting a particular nucleic acid sequence in a test sample, comprising

(a) immobilizing the nucleic acid sequence to be detected to a solid phase,

(b) forming a complex between the particular nucleic acid sequence to be detected in the sample and a sequence of PNA that is complementary to the nucleic acid sequence to be detected, the complex having at least one epitope for an antibody according to any of the claims 1 to 7,

(c) contacting any complex that is formed between the PNA sequence and the nucleic acid sequence to be detected with an antibody according to any of the claims 1 to 7, and

(d) determining the presence of antibody-PNA-nucleic acid complexes.

13. A kit for detecting a particular nucleic acid sequence in a sample, said kit containing antibody according to any of the claims 1 to 7, a PNA sequence that is complementary to the nucleic acid sequence to be detected and a detection system.

INTERNATIONAL SEARCH REPORT

International Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K16/44 G01N33/577 G01N33/53 G01N33/543

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K G01N C12Q C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NATURE, vol.365, no.6446, 7 October 1993, LONDON, GB pages 566 - 568 M. EGHOLM ET AL. 'PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules.' cited in the application see the whole document --- -/--	1-13

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

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Date of the actual completion of the international search	Date of mailing of the international search report
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF THE CHEMICAL SOCIETY. D. CHEMICAL COMMUNICATIONS, no.9, 1993, LONDON, GB pages 800 - 801 M. EGHOLM ET AL. 'Peptide nucleic acids containing adenine and guanine recognize thmine and cytosine in complementary DNA sequences.' cited in the application see the whole document ---	1-13
A	JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol.114, no.5, 1992, WASHINGTON DC, USA pages 1895 - 1897 M. EGHOLM ET AL. 'Peptide nucleic acids (PNA). Oligonucleotide analogs with an achiral peptide backbone.' cited in the application see the whole document ---	1-13
A	TRENDS IN BIOTECHNOLOGY, vol.11, no.9, September 1993, CAMBRIDGE, GB pages 384 - 386 O. BUCHARDT ET AL. 'Peptide nucleic acids and their potential applications in biotechnology.' see the whole document ---	1-13
A	SCIENCE, vol.254, no.5037, 6 December 1991, WASHINGTON DC, USA pages 1497 - 1500 P. NIELSEN ET AL. 'Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide.' see the whole document ---	1-13
P,A	BIOCONJUGATE CHEMISTRY, vol.5, no.1, January 1994, WASHINGTON DC, USA pages 3 - 7 P. NIELSEN ET AL. 'Peptide nucleic acid (PNA). A DNA mimic with a peptide backbone.' see the whole document -----	1-13

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